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Variable number of tandem repeats of 9 *Plasmodium vivax* genes among Southeast Asian isolates

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ABSTRACT

The variable number of tandem repeats (VNTRs) provides valuable information about both the functional and evolutionary aspects of genetic diversity. Comparative analysis of 3 *Plasmodium falciparum* genomes has shown that more than 9% of its open reading frames (ORFs) harbor VNTRs. Although microsatellites and VNTR genes of *P. vivax* were reported, the VNTR polymorphism of genes has not been examined widely. In this study, 230 *P. vivax* genes were analyzed for VNTRs by SERV, and 33 kinds of TR deletions or insertions from 29 *P. vivax* genes (12.6%) were found. Of these, 9 VNTR fragments from 8 *P. vivax* genes were used for PCR amplification and sequence analysis to examine the genetic diversity among 134 isolates from four Southeast Asian countries (China, Republic of Korea, Thailand, and Myanmar) with different malaria endemicity. We confirmed the existence of extensive polymorphism of VNTR fragments in field isolates. This detection provides several suitable markers for analysis of the molecular epidemiology of *P. vivax* field isolates.

1. Introduction

Plasmodium vivax is a relatively neglected human malaria parasite despite representing a major public health challenge for Southeast Asia, the Western Pacific, the Middle East, and South and Central America, where 2.85 billion people are currently at risk of infection and 70–80 million clinical cases are reported each year (Guerra et al., 2010; Mueller et al., 2009). It could be argued that *P. vivax* is more difficult to control, eliminate and eradicate than *P. falciparum*; thus, it constitutes a hurdle for national strategies for control and elimination (Galinski and Barnwell, 2008; Mueller et al., 2009; Talha et al., 2015). Therefore, to eradicate malaria, further studies on the genetic diversity, population structure, and transmission of plasmodium parasites in various endemic areas are needed to provide essential information for disease control and management. Understanding the genetic structure of *P. vivax* is important for accurately describing the transmission dynamics of vivax malaria. These data are also essential for developing, designing and evaluating mitigation strategies. Moreover, the malaria parasite population structure has a significant impact on the organism's gene flow and the rate of emergence and spread of new mutations, which can result in drug resistance or vaccine-induced immunity inhibition (De Brito and Ferreira, 2011; Talha et al., 2015).

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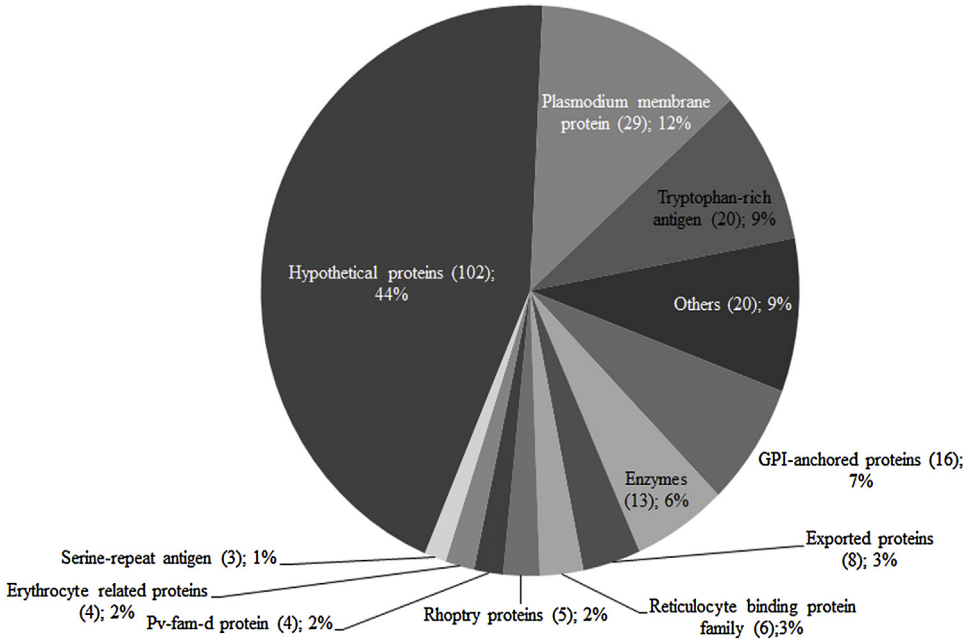

Fig. 1. Classification of the putative functional proteins of 230 *Plasmodium vivax* genes for the VNTR sequence analysis. Parentheses indicate the number of genes.

Molecular markers which have been used to examine *P. vivax* population diversity can be classify into two groups: (1) microsatellites and (2) variable number of tandem repeats (VNTRs) genes. The most widely used microsatellites markers are a set of 14 polymorphic microsatellites described by Karunaweera et al. (2007), which were used to analyze the population structures of *P. vivax* in Brazil, Vietnam, Sri Lanka, Myanmar, Ethiopia, Peru and ROK (Ferreira et al., 2007; Gunawardena et al., 2010; Iwagami et al., 2012; Karunaweera et al., 2007, 2008; Van den Eede et al., 2010, 2011). While, current VNTR markers including genes encoding merozoite surface protein 1 (*msp-1*), *msp-3α*, and *msp-3β* (Bruce et al., 1999; Putaporntip et al., 1997; Zakeri et al., 2006), circumsporozoite protein (*csp*) (Imwong et al., 2005; Qari et al., 1994), apical membrane antigen-1 (*ama-1*) (Figtree et al., 2000), Duffy binding protein (*dbp*) (Xainli et al., 2000). VNTRs can produce duplicate protein domains (Verstrepen et al., 2005) or affect protein expression level and transcription (Pagani et al., 2000; Vinces et al., 2009; Whetstine et al., 2002). VNTRs provide valuable information on both the function of proteins in the humoral immune response and the evolutionary aspects of genetic diversity in the protozoan parasites (Coil et al., 2008; Goto et al., 2008, 2006; Schofield, 1991; Tan et al., 2010). Proteins containing TRs have been observed in many protozoan parasites, such as *P. falciparum* (Coppel et al., 1984; Dame et al., 1984; Koenen et al., 1984), *Leishmania* (Goto et al., 2006; Goto et al., 2007), *Trypanosoma* species (Goto et al., 2008) and the helminth *Schistosoma japonicum* (Angeles et al., 2011), and have been used to evaluate antigenicity during infection by these pathogens. Comparative analysis of 3 *P. falciparum* genomes showed that more than 9% of *P. falciparum* ORFs are VNTRs (Tan et al., 2010). However, the VNTR polymorphism of *P. vivax* ORFs has not been examined widely.

The purpose of this study was to identify some useful *P. vivax* VNTRs markers and use them to study genetic diversity from Southeast Asian (SEA) malaria-endemic countries. A total of 230 *P. vivax* genes were selected and subjected to polymerase chain reaction (PCR) amplification and sequencing. The VNTRs were analyzed by sequence-based estimation of repeat variability (SERV)(Legendre et al., 2007). Nine TR sequences of *P. vivax* that were suitable for genotyping purposes (i.e., a VARscore in the SERV analysis of 1–3) were selected and used as markers to examine the prevalence and extent of these TR sequences among 134 isolates from Southeast Asian (SEA) malaria-endemic countries.

2. Materials and methods

2.1. Selection of candidates

The putative functional proteins of 230 *Plasmodium vivax* genes for the VNTR sequence analysis were classified (Fig. 1). The malaria parasite information was derived from the functional genomic databases PlasmoDB (http://plasmodb.org/plasmo) and Tropical Disease Research (TDR) Targets (http://tdrtargets.org). The candidate sources were from i. well-characterized *P. vivax* genes/proteins, such as merozoite surface protein (MSP), rhoptry protein, reticulocyte binding protein (RBP), and serine repeat antigens (SERA); ii. orthologs of *P. falciparum* immunogenic proteins (Crompton et al., 2010); and iii. proteins with signal peptide (SP) and 1–3 transmembrane domains (TM). Because of the difficulty of obtaining the ORFs of candidates, the genes with multiple exons were BLAST-searched in the expressed sequence tags (ESTs) database to identify targets in the cDNA library (Cui et al., 2005), and only the ORFs that could be amplified from the library were selected.

2.2. Origin of blood samples and DNA extraction

The Korean patients' samples ($n = 38$) were collected from patients with symptoms and positive *P. vivax* parasitemia, as assessed by microscopic examination at local health centers and clinics in Gangwon and Gyeonggi Provinces in the Republic of Korea (ROK) from 2010. The Thai sample (THA, $n = 32$) obtained from symptomatic, smear-positive patients in Mae Sod, Thailand. The samples from Myanmar (MM, $n = 32$) were collected in 2012 from patients from the Shwe Kyin area of Myanmar who were confirmed to be positive for vivax malaria by a malaria antigen rapid test (FK80; Standard Diagnostics, Gyeonggi, ROK) and microscopic examination. The samples from China were kindly supplied by the JIPD, Wuxi, Jiangsu Province of China. Genomic DNA was extracted from 200 μL of whole blood samples from a *P. vivax* patient using a QIAamp DNA Blood Mini Kits (Qiagen, Valencia, CA, USA), which